Glaucoma

COCH Transgene Expression in Cultured Human Trabecular Meshwork Cells and Its Effect on Outflow Facility in Monkey Organ Cultured Anterior Segments

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Purpose. To determine the effects of *COCH* transgene expression on cultured human trabecular meshwork (HTM) cell morphology and on outflow facility (OF) in monkey organ cultured anterior segments (MOCAS).

Methods. An adenoviral (Ad) vector expressing both cochlin (*COCH*) and green fluorescent protein (GFP) (Ad*COCH*GFP) or GFP alone (AdGFP) was used to transduce cultured HTM cells (multiplicity of transduction, 2.8 and 28). *COCH* transgene expression in transduced HTM cells and the culture medium was verified by Western blot analysis and immunofluorescence detection 5 days after transduction. MOCAS were used to test the effect of Ad vectors (2.8×10^{10} viral particles per segment) on OF. The morphology of transduced MOCAS was evaluated by light microscopy.

Results. Western blot analysis showed a viral vector dose-dependent expression of cochlin in transduced cells and the culture medium. There was no notable morphologic change in transduced cells. In MOCAS, cochlin expression was detectable in the medium by 3 days after transduction. A 35% decrease in OF in Ad*COCH*GFP-transduced MOCAS was detected after 3 days, decreasing by 76% after 12 days when compared to control segments injected with AdGFP. Anterior segment pressure (ASP) more than doubled ($P < 0.05$) in segments injected with Ad*COCH*GFP at 12 days after transduction. Light microscopy revealed normal angle structures in transduced segments.

Conclusions. Ad vector delivery of the *COCH* transgene resulted in cochlin expression in HTM cells and MOCAS. Cochlin expression was effective in decreasing OF and increasing ASP in MOCAS, suggesting possible involvement of cochlin in IOP elevation in vivo. *COCH* gene delivery has potential for use in developing a glaucoma model. (*Invest Ophthalmol Vis Sci.* 2010;51:2060–2066) DOI:10.1167/iovs.09-4521

Glaucomas are a group of irreversible blinding eye diseases. The projected number of people affected with glaucoma worldwide in 2010 is approximately 60.5 million.[1] Primary open-angle glaucoma (POAG) is the most common form of glaucoma, where intraocular pressure (IOP) is a causal risk factor for optic neuropathy and lowering IOP has beneficial effects.[2–5] Elevation in IOP occurs because of an imbalance between aqueous humor production and outflow.[6] This imbalance occurs in part in glaucomatous eyes because of an increased resistance to aqueous humor outflow, particularly within the trabecular meshwork (TM) of the anterior segment. Changes within the extracellular matrix (ECM) of the TM, especially the increased deposition of ECM proteins, are thought to contribute to the increased resistance to outflow.[7]

Cochlin is an ECM protein[8] that was identified in human glaucomatous but not in control TM tissue by proteomic analysis and was associated with TM protein and mucopolysaccharide deposits.[9] It is also expressed in glaucomatous DBA/2J mice but not in wild-type (CD1 or C57BL6J) mice.[10] Structurally, cochlin contains a signal peptide for secretion into the ECM,[8] a factor C homology (FCH) domain, and two von Willebrand factor A-like (vWFA) domains.[11,12] It undergoes multimerization in response to applied shear stress,[9] which is a property of the vWF domain.[13] Cochlin deposits are also found in the inner ear where it is associated with progressive hearing loss disorder DFNA9.[8,14] Glaucoma and autosomal dominant nonsyndromic sensorineural hearing loss (DFNA9) both are late-onset and progressive diseases and are also associated with local changes in fluid flow: glaucoma with fluid flow in the TM and DFNA9 with fluid flow in the inner ear.[15,16]

In the TM, cochlin is secreted into the ECM.[15] Its expression in anterior segment organ culture models is increased after TGFβ2-treatment[17] and is associated with anterior segment pressure (ASP) elevation and outflow facility (OF) reduction. Whether cochlin overexpression alone is sufficient to elevate IOP (in the anterior segment culture context: ASP) or is a consequence of other TGFβ2-induced changes relevant to IOP elevation is not yet known. Injection of siRNA against cochlin in the New Zealand White rabbits decreases IOP by approximately 25% for 78 hours, suggesting that cochlin expression can influence IOP (Graham FL, et al. *IOVS* 2007;48:ARVO E-Abstract 4808). We have shown that exogenous cochlin also results in aggregation of isolated primary TM cells.[9]

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Supported by National Institutes of Health Grants R01 EY002698, R01 EY017006, R01 EY016236, P30 EY016665, P51 RR000167, S10 RR019382, P30 EY01480, R03 EY15266, and R01 EY016112; unrestricted grants from Research to Prevent Blindness (RPB); an RPB Career Award (SKB); a faculty research grant 6-2009-0515 from Yonsei University College of Medicine for 2009 (ESL), and funds from the Ocular Physiology Research and Education Foundation (PLK).

Submitted for publication August 20, 2009; revised October 28, 2009; accepted November 4, 2009.

Disclosure: **E.S. Lee**, None; **B.T. Gabelt**, None; **J.A. Faralli**, None; **D.M. Peters**, None; **C.R. Brandt**, None; **P.L. Kaufman**, None; **S.K. Bhattacharya**, None

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In the present study, we determined whether adenovirus-mediated expression of cochlin alone is sufficient to alter the morphology of cultured human trabecular meshwork (HTM) cells and/or alter ASP and OF in monkey organ cultured anterior segments (MOCAS).

METHODS

Construction of Recombinant Replication-Deficient Adenoviral Vector Carrying Human Cochlin and GFP Genes

An adenoviral vector construction system (AdEasy Adenoviral system; Stratagene, La Jolla, CA) was used. Cochlin (*COCH*) cDNA (GenBank accession number AF006740; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) was subcloned into the shuttle vector, pShuttle-CMV, with an influenza virus hemagglutinin (HA) tag fused to the carboxyl terminus of *COCH*. This dual-expression vector also contained the gene for GFP downstream from an internal ribosome entry site (IRES) element, so that *COCH* and GFP could be expressed from the same transcript. The resultant pShuttle-*COCH* was then used to generate adenoviral recombinants through homologous recombination with the adenoviral backbone vector, pAdEasy-1, in BJ5183 bacterial cells (Stratagene). For packaging, the *COCH* vector DNA was linearized by digestion with *Pac*I. The linearized DNA was then transfected into HEK293 cells (Lipofectamine Plus; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cultures were transfected at 37°C with 5% CO_2 for 3 hours, at which time the medium was replaced with DMEM with 10% fetal bovine serum (FBS). A separate adenovirus vector expressing GFP alone (AdGFP) was used as the control. Virus titers were determined by spectrophotometric absorbance at 260 nm of viral particles (VPs), as described previously.[18]

Transduction of HTM Cells

HTM cells were isolated, as previously described.[19–21] from a 17-year-old male donor and a 27-year-old female donor, with no known history of ocular disease. All experiments were performed with cultures between passages 5 and 7. The cells were grown to confluence in low-glucose DMEM (Sigma-Aldrich, St. Louis, MO) containing 15% FBS, 2 mM L-glutamine, 1% amphotericin B (Mediatech, Herndon, VA), 0.05% gentamicin (Mediatech), and 1 ng/mL FGF-2 (Peprotech, Rocky Hill, NJ). Cultures were maintained for one additional week after confluence, after which time FGF-2 was removed from the medium and the serum level reduced to 10%. All the cultures were then used within 1 to 3 days and exhibited a stable endothelial-like morphology at the time when they were transduced with AdGFP or Ad*COCH*GFP. The cultures were transduced at multiplicities of transduction (MOT) of 2.8 and 28 in DMEM containing 10% FBS and 2 mM L-glutamine. Controls were nontransduced cells that were incubated with fresh DMEM containing 10% FBS.

Morphology of HTM Cell Cultures Transduced with Adenovirus Vectors

Light microscope images of transduced cultures were captured with an inverted microscope (Axiovert 200; Carl Zeiss Meditec, Inc., Thornwood, NY) equipped with a high-resolution color camera (AxioCam; Carl Zeiss Meditec, Inc.), on the day of and 5 days after transduction All images were processed with the system software (Axiovision 4.2; Carl Zeiss Meditec, Inc.).

Western Blot Analysis

To confirm expression of the cochlin protein, both HTM cell lysates and medium were collected 5 days after transduction. The HA-cochlin in the medium was isolated by immunoprecipitation with anti-HA Sepharose (Covance, Emeryville, CA), collected by centrifugation, and resuspended in Laemmli sample buffer[22] containing 2% β-mercaptoethanol. Cell lysates were prepared by scraping the cells from culture dishes into 1× phosphate-buffered saline (PBS). Microscopic examination confirmed that >99.99% of the cells were removed from the dishes. The cells were then pelleted by centrifugation at 2500 rpm for 3 minutes and resuspended in sample buffer containing 2% β-mercaptoethanol. The cells were lysed by sonication for 12 cycles on ice. Both cell lysates and medium were boiled for 5 minutes and were electrophoresed in 10% SDS-polyacrylamide gels. The proteins were then electrophoretically transferred to nitrocellulose, blocked with 5% nonfat dry milk in 1× buffer (Genius buffer I; 100 mM maleic acid, 150 mM NaCl [pH 7.5]; Techne, Princeton, NJ) with 0.3% vol/vol Tween-20 and probed with polyclonal anti-cochlin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was washed and incubated with secondary donkey anti-goat IgG conjugated with horseradish peroxidase (HRP; Santa Cruz Biotechnology) and then developed with the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ), according to the manufacturer's instructions and exposed to x-ray film.

Cochlin expression in the medium from MOCAS was also analyzed by Western blot analysis. For these studies medium was collected overnight on days 0, 3, 6, 9, and 12 (one sample was collected at 11 days) and in some cases 24 days after transduction. All medium/perfusate was removed from the dish on the day before the collection day. On the designated collection day, the medium was aspirated into a syringe, transferred to a tube, and frozen at −70°C until it was analyzed. The average ± SEM duration of the medium collection period was 24 ± 2 hours. The HA-cochlin in medium was isolated by immunoprecipitation, as described for the cell culture medium.

ASP and OF in MOCAS

Eyes from rhesus monkeys (*Macaca mulatta*) and cynomolgus monkeys (*Macaca fascicularis*) from either the Wisconsin National Primate Research Center or Dr. Kaufman's colony at the University of Wisconsin School of Medicine and Public Health, which were being euthanatized for other protocols (adhering to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research) were obtained fresh and placed in organ culture, usually less than 2 hours after enucleation. The anterior segments were prepared for organ culture as described in detail in a previous report.[23] Briefly, the eyes were bisected at the equator. The lens, iris, and ciliary body were removed from the anterior segment. The anterior segment was then mounted in the organ culture dish and secured with tissue glue and a plastic ring lined with aquarium silicone. Medium was infused at a constant rate of 2.5 μL/min except when OF was being measured. Baseline (BL) OF was measured after overnight equilibration by using the two-level constant pressure perfusion technique.[24] Briefly, fluid flow from an external reservoir was measured for approximately 1 hour, alternating every 4 minutes between two different pressures 10 mm Hg apart. OF was calculated as the change in flow divided by the change in pressure and sequentially averaged. Medium infusion continued at a constant rate of 2.5 μL/min when OF was not being measured. ASP in MOCAS was monitored (Isotec transducers/HSE Tam-D amplifier; Harvard Apparatus, Holliston, MA) and recorded every 15 minutes throughout the experiment, except at those times when infusion was temporarily stopped to take OF measurements or to replenish the medium in the infusion syringe (roughly once every 72 hours). It is important to note that we have compared the change after adjusting BL values; therefore the differences in BL between test and control groups are not expected to affect the measurable outcome. Anterior segments were injected via the inflow port with ~80 μL containing 2.8×10^{10} VPs of Ad*COCH*GFP, whereas the contralateral anterior segments received the same dose of AdGFP. Studies were usually terminated after 12 days but in some cases were maintained for up to 24 days.

GFP Expression and Cochlin Localization in the TM of MOCAS

At the endpoint of the cochlin OF studies, all MOCAS were perfused at ~8 mm Hg with 5 mL of 4% paraformaldehyde for 30 minutes, cut into

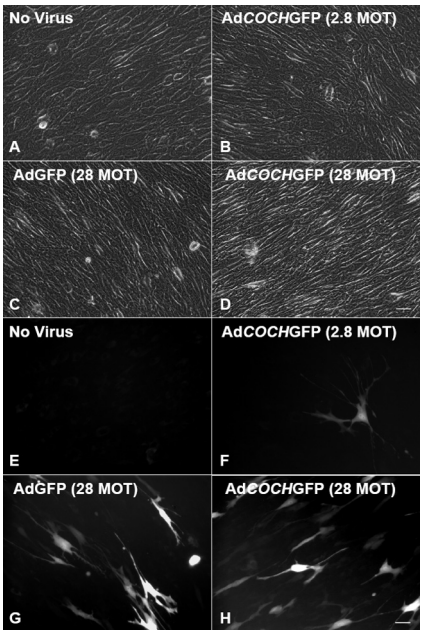

FIGURE 1. Human trabecular meshwork cell morphology and GFP expression 5 days after transduction with adenoviral vectors. No alteration in trabecular meshwork cell morphology was apparent after transduction with Ad*COCH*GFP or AdGFP (**A–D**). GFP expression was examined by inverted microscopy (**E–H**). (**A**, **E**) Nontransduced control. (**B**, **D**, **F**, **H**) Ad*COCH*GFP transduced at MOTs of 2.8 (**B**, **F**) and 28 (**D**, **H**). (**C**, **G**) AdGFP transduced at MOTs of 28. Bar, 50 μm.

quadrants or eighths, and then immersed in 4% paraformaldehyde. A piece from each quadrant of each MOCAS was examined by fluorescence microscopy for GFP fluorescence in the outflow pathways and then embedded in paraffin, and 5-μm sections were prepared. To assess the integrity of angle structure and Schlemm's canal, we stained sections with hematoxylin and eosin (H&E). To localize cochlin in transduced segments, sections were mounted on glass slides, deparaffinized, and blocked in 0.2% bovine serum albumin (BSA; Sigma-Aldrich)/PBS after previously described procedures.[9] Sections were labeled with a 1:50 dilution of goat polyclonal anti-cochlin antibody[15] for 1 hour at room temperature followed by Alexa 594-conjugated donkey anti-goat IgG (1:300 dilution, 1 hour; Invitrogen). Sections were also labeled with Hoechst 33342 (cat. no. H1399; Invitrogen) to visualize nuclei.

Data Analysis

ASP and OF data are expressed as the mean ± SEM and were analyzed by the two-tailed paired *t*-test for ratios compared to 1.0 (OF) or for differences compared with 0.0 (ASP). For data analysis, only segments demonstrating GFP expression and BL OF of less than 0.75 μL/min/mm Hg were included. Mean ASP ± SEM for the designated time points was usually calculated by averaging 40 consecutive pressure measurements at least 2 hours after any system stoppage. For unpaired group analysis, all segments were analyzed by the two-tailed unpaired *t*-test. For paired group analysis, only contralateral anterior segments from a given monkey were compared by the two-tailed paired *t*-test (all analyses: SPSS 11.5; SPSS, Chicago, IL). Ad*COCH*GFP and AdGFP transduced segments were compared with each other after normalization for BL differences. Ad*COCH*GFP segments were defined as responders when an OF decrease of at least 50% was observed at 12 days after Ad*COCH*GFP transduction after normalization for BL. Repeated-measures ANOVA (RMANOVA) was performed to evaluate the time-course of OF responses for Ad*COCH*GFP and AdGFP.

RESULTS

COCH Transgene Expression in Cultured HTM Cells

HTM cells transduced with Ad*COCH*GFP at either MOT 2.8 or 28 did not show any morphologic changes compared with nontransduced or AdGFP transduced control cells transduced at MOT 28 (Figs. 1A–D). The morphology of AdGFP-transduced cells at the lower MOT (2.8) was also unchanged compared with nontransduced cells (data not shown). Transduction of HTM cells by AdGFP or Ad*COCH*GFP was confirmed by GFP expression (Figs. 1E–H). Western blot analysis also confirmed the expression of cochlin and showed a viral vector dose-dependent expression of a ~60- to 66-kDa protein corresponding to cochlin 5 days after transduction in Ad*COCH*GFP-transduced cells and medium but not in nontransduced or AdGFP-transduced cells or medium (Fig. 2). Lysates from cells transduced with Ad*COCH*GFP also showed a ~40-kDa isoform band of cochlin.[8,25] Lower molecular weight bands in the Ad*COCH*GFP transduced cells (shown) and medium (not shown) detected by anti-cochlin antibody may represent cochlin degradation products.[8,26]

Cochlin Expression in MOCAS

At the conclusion of the experiment, GFP fluorescence in the MOCAS was examined (Fig. 3) to confirm the efficient transduction by either Ad*COCH*GFP or AdGFP. Although most GFP expression occurred in the TM (Fig. 3), occasional fluorescence was also visible in the corneal endothelium and the remnant of the ciliary body. Cochlin expression was also confirmed by using anti-cochlin staining in paired anterior segments transduced with AdGFP (Figs. 3A–D) or Ad*COCH*GFP (Figs. 3E–H). The paired control segments, transduced with AdGFP, showed prominent GFP expression (Fig. 3B) but failed to demonstrate any positive staining for cochlin (Fig. 3C). In contrast, the TM of anterior segments transduced with Ad*COCH*GFP demonstrated strong, positive staining for cochlin (Fig. 3G) in areas showing GFP expression (Fig. 3F), indicating robust cochlin expression.

Tissue cellularity was similar for segments transduced with AdGFP or Ad*COCH*GFP (Figs. 3A, 3E) as demonstrated by the presence of cell nuclei (Figs. 3D, 3H), an intact Schlemm's canal and organized trabecular beams in both AdGFP and Ad*COCH*GFP transduced segments (Fig 3).

Transduction of the *COCH* gene with adenoviral vectors also resulted in the secretion of the expressed cochlin into the medium (Fig. 4). Peak cochlin secretion into the medium was

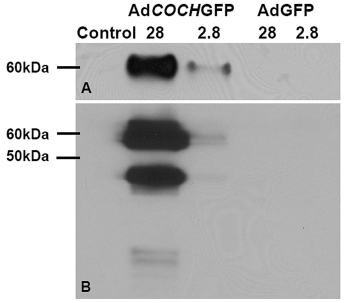

FIGURE 2. Western blot analysis for cochlin demonstrating viral vector dose-dependent cochlin expression in Ad*COCH*GFP-transduced cells. Nontransduced cells served as the control. The cells were transduced with either AdGFP or Ad*COCH*GFP at MOTs of 28 or 2.8, as indicated. Cell lysates and medium were collected 5 days after transduction. (**A**) Medium and (**B**) cell lysates probed with anti-cochlin antibody after transfer to a PVDF membrane.

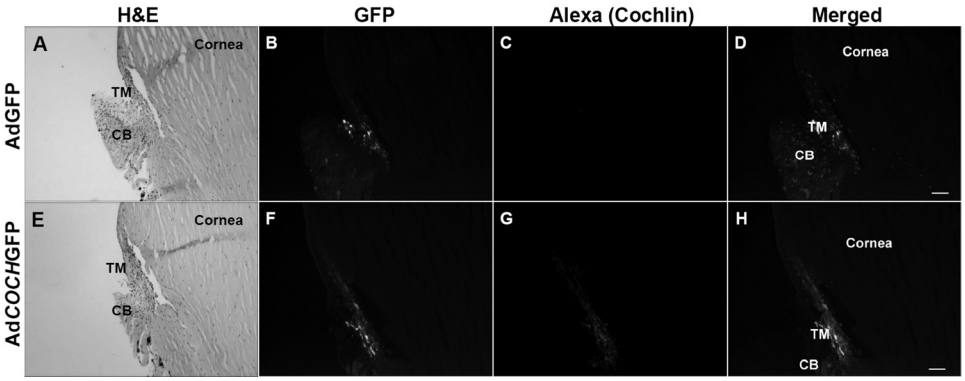

FIGURE 3. Cochlin localization in Ad*COCH*GFP-transduced MOCAS. (**A–D**) AdGFP-transduced segment. (**E–H**) Ad*COCH*GFP-transduced segment. The hematoxylin-eosin–stained anterior eye segments (H&E) are shown to demonstrate cellularity. Paraffin-embedded sections (5 μm) showed GFP expression (*green* channel; **B**, **F**) and were labeled for cochlin (*red* channel, Alexa Fluor; **C**, **G**). Merged images including DAPI-stained cell nuclei (*blue* channel) are shown in (**D**) and (**H**). Note that only segments transduced with Ad*COCH*GFP showed cochlin (**G**). Bar, 50 μm.

detected 3 days after transduction which was the earliest time point examined. Cochlin expression persisted for up to 24 days (data not shown) after transduction, although the level of expression in the medium was weaker than that observed at 3 days. Cochlin was absent in medium collected before transduction or in medium from AdGFP-transduced segments (Fig. 4).

Effect of Cochlin Expression on OF in MOCAS

Only segments that had normal BL OF in the range of 0.20 to 0.75 μL/min/mm Hg were considered. Nine Ad*COCH*GFP- and seven AdGFP-transduced segments originating from nine rhesus and two cynomolgus monkeys were included for data analysis. Of these 16 segments, there were five in vivo contralateral pairs. Table 1 shows the effect of Ad-transduced expression of cochlin in MOCAS on OF. Unpaired and paired analysis showed that BL OF for Ad*COCH*GFP (0.44 ± 0.04 and 0.42 ± 0.05) was lower than that of AdGFP (0.52 ± 0.05 and 0.56 ± 0.05) segments (both $P < 0.01$). The analysis showed that a reduction in OF in Ad*COCH*GFP was detected 3 days after transduction (35% decrease, unpaired, $P < 0.001$) compared with AdGFP segments after a correction for BL facility. OF decreased further over the 12-day duration of the study (76% decrease, unpaired, $P < 0.001$). A similar trend was found when paired segments were analyzed. In this instance, a 55% reduction occurred at 12 days after transduction ($P < 0.05$). When only responder Ad*COCH*GFP segments were included, OF was decreased by 83% and 68%, for unpaired ($n = 7$) and paired analysis ($n = 4$), respectively, 12 days after transduction. Time course analysis for the OF response after transduction revealed that, whereas OF was not changed in AdGFP-transduced segments, OF in Ad*COCH*GFP-transduced segments was significantly decreased 9 to 12 days after transduction when compared to BL (RMANOVA, unpaired segments, Fig. 5, $P = 0.001$; paired segments, data not shown, $P = 0.022$).

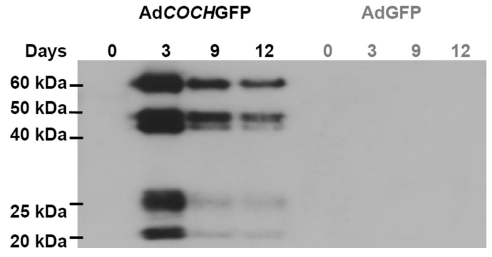

FIGURE 4. Western blot analysis demonstrating cochlin secretion into the medium of Ad*COCH*GFP-transduced MOCAS. Detection was performed with an antibody specific to cochlin protein after immunoprecipitation of the medium by anti-HA Sepharose. Ad*COCH*GFP- and AdGFP-transduced MOCAS media were subjected to analysis as indicated.

Change in ASP was evaluated in a similar manner. Although BL ASP before adenovirus injection was not significantly different between AdGFP-and Ad*COCH*GFP-transduced segments, ASP posttransduction in Ad*COCH*GFP-transduced segments was elevated by approximately 140% to 170% (unpaired and paired, $P < 0.05$) by day 12 when compared with AdGFP-transduced segments after correction for BL. There was no detectable ASP increase in two segments (Fig. 6), and so the results were also evaluated using only responder segments. When the responder segments were evaluated, the magnitude of ASP increase in Ad*COCH*GFP-injected segments was approximately 150% to 210% (unpaired and paired, $P < 0.05$). The two pairs of segments that did not demonstrate any ASP elevation after transduction (Fig. 6) had detectable levels of cochlin in the tissue and medium.

DISCUSSION

This study demonstrated, for the first time, that cochlin expression alone results in an OF decrease and subsequent ASP increase. Previously,[17] we demonstrated that TGFβ2-induced ASP elevation was associated with an increase in cochlin secretion into the medium and expression in the tissue of monkey and pig organ-cultured anterior segments treated with TGFβ2. Since TGFβ2 has been shown to increase TM ECM protein production leading to ECM remodeling, previously reported reductions in the outflow of aqueous humor and elevation in ASP may be attributable to the production of these other proteins.[27–29] Whether the increase in IOP in human and DBA/2J mice[9] as well as in the organ culture systems is due solely to the overexpression of cochlin or interactions between cochlin and other proteins in the ECM is still unknown.

The magnitude and time of onset of the ASP change in seven responders was quite variable despite the fact that all segments in this study were injected with a fixed amount of virus (2.8×10^{10} VPs/segment; Fig. 6) and cochlin was consistently expressed (data not shown). In five of the nine MOCAS that showed GFP and cochlin expression, the ASP increase was gradual, whereas two responders (segments 1 and 3, Fig. 6) showed a decrease in OF with subsequent ASP increase after day 9. The gradual change in ASP suggests that the effect may be due to the time it took to reach a critical level of cochlin expression in the TM. This notion would be consistent with the fact that the maximum secretion of cochlin into the medium occurred at 3 days after Ad*COCH*GFP transduction.

Alternatively, the variability in the responses may be because the efficiency of vector delivery and transduction in organ culture and/or the tissue susceptibility varied between segments. Several factors govern gene expression from a viral vector, including local fluid dynamics at the time of injection and intrinsic properties of the host tissue, not all of which are

TABLE 1. Effect of Cochlin on Outflow Facility of MOCAS

	Unpaired Segments			Paired Segments		
	Outflow Facility			Outflow Facility		
	Ad*COCH*GFP	AdGFP	Ad*COCH*GFP/AdGFP	Ad*COCH*GFP	AdGFP	Ad*COCH*GFP/AdGFP
All						
BL	0.44 ± 0.04	0.52 ± 0.05	0.84 ± 0.11*	0.42 ± 0.05	0.56 ± 0.05	0.75 ± 0.04*
Rx(d3)	0.40 ± 0.06	0.71 ± 0.08	0.57 ± 0.11†	0.36 ± 0.05	0.72 ± 0.10	0.52 ± 0.07*
Rx(d3)/BL	0.91 ± 0.11	1.40 ± 0.15	0.65 ± 0.11†	0.90 ± 0.15	1.33 ± 0.21	0.71 ± 0.11
Rx(d6)	0.35 ± 0.07	0.65 ± 0.14	0.54 ± 0.15†	0.31 ± 0.10	0.62 ± 0.17	0.59 ± 0.18
Rx(d6)/BL	0.81 ± 0.18	1.28 ± 0.25	0.63 ± 0.19†	0.78 ± 0.31	1.17 ± 0.31	0.79 ± 0.24
Rx(d9)	0.27 ± 0.06	0.72 ± 0.21	0.38 ± 0.14†	0.23 ± 0.07	0.69 ± 0.26	0.47 ± 0.17‡
Rx(d9)/BL	0.63 ± 0.15	1.41 ± 0.36	0.45 ± 0.16†	0.55 ± 0.21	1.29 ± 0.45	0.61 ± 0.21
Rx(d12)	0.15 ± 0.04	0.81 ± 0.26	0.19 ± 0.07†	0.16 ± 0.06	0.69 ± 0.28	0.35 ± 0.14*
Rx(d12)/BL	0.37 ± 0.10	1.53 ± 0.46	0.24 ± 0.10†	0.39 ± 0.17	1.30 ± 0.49	0.45 ± 0.16‡
Responder						
BL	0.46 ± 0.05	0.58 ± 0.04	0.79 ± 0.11*	0.44 ± 0.06	0.59 ± 0.05	0.73 ± 0.04*
Rx(d3)	0.38 ± 0.07	0.75 ± 0.10	0.51 ± 0.12†	0.33 ± 0.04	0.72 ± 0.13	0.48 ± 0.08*
Rx(d3)/BL	0.81 ± 0.11	1.32 ± 0.20	0.61 ± 0.30†	0.77 ± 0.11	1.24 ± 0.23	0.68 ± 0.14
Rx(d6)	0.28 ± 0.06	0.71 ± 0.19	0.39 ± 0.13†	0.22 ± 0.05	0.62 ± 0.22	0.46 ± 0.17‡
Rx(d6)/BL	0.58 ± 0.09*	1.28 ± 0.35	0.45 ± 0.14*	0.48 ± 0.10	1.10 ± 0.39	0.64 ± 0.24
Rx(d9)	0.21 ± 0.06	0.86 ± 0.28	0.25 ± 0.10†	0.17 ± 0.05	0.73 ± 0.33	0.37 ± 0.17‡
Rx(d9)/BL	0.45 ± 0.11†	1.41 ± 0.37	0.45 ± 0.16†	0.36 ± 0.11	1.31 ± 0.57	0.49 ± 0.22
Rx(d12)	0.12 ± 0.02	0.88 ± 0.31	0.14 ± 0.05†	0.11 ± 0.04	0.75 ± 0.35	0.24 ± 0.11*
Rx(d12)/BL	0.28 ± 0.05	1.62 ± 0.55	0.17 ± 0.06†	0.24 ± 0.08	1.35 ± 0.62	0.32 ± 0.14‡

Data are the mean ± SEM. Ad*COCH*GFP to AdGFP ratio (Ad*COCH*GFP/AdGFP) was significantly different from 1.0 by the two-tailed, two-sample *t*-test for unpaired segments or by the paired *t*-test for paired segments as indicated. For unpaired segments: all, $n = 9$ for Ad*COCH*GFP (8 for day 12); $n = 7$ for AdGFP (6 for day 12); responder, $n = 7$ for Ad*COCH*GFP; $n = 5$ for AdGFP. For paired segments: all, $n = 5$ (5 contralateral pairs included in the unpaired 16 segments); responder, $n = 4$ (5 contralateral pairs included in the unpaired 12 responder segments). BL, baseline; Rx, treatment with adenovirus as indicated.

* $P < 0.01$.

† $P < 0.001$.

‡ $P < 0.05$.

well understood. Relatively fresh tissue often appears to possess greater viral load tolerance and greater expression levels compared with older tissue as exemplified by comparing different reports.[30–34] In one study, approximately 3×10^{10} VPs were well tolerated in vivo and showed consistent expression for more than 2 years,[32] while in another study, 10^8 pfu ($\sim 10^{10}$ VP) caused 100% loss of TM cells in human organ culture.[31] The levels of transduction obtained in organ culture with adenoviral vectors may vary experimentally, and this could be a factor in the variability of the results observed.

In studies conducted with MOCAS treated with TGFβ2 as well as in the current studies of cochlin overexpression, ASP elevation required some time to develop. However, in both experiments, the time-dependent reduction in OF was approx-

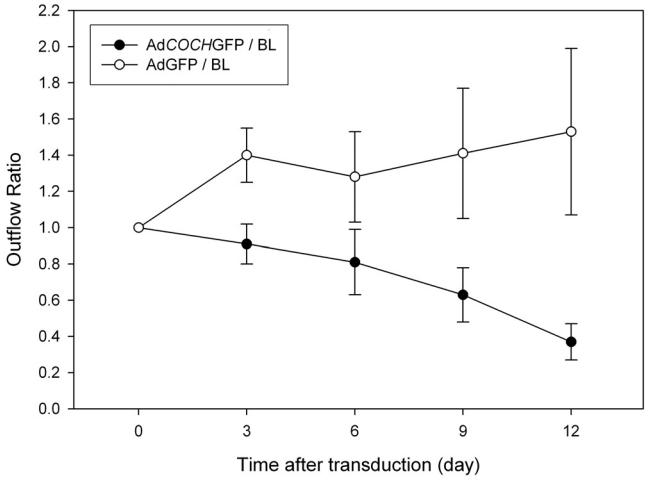

FIGURE 5. Time-course of OF response normalized to that of BL for Ad*COCH*GFP ($n = 8$)- and AdGFP ($n = 6$)-transduced segments. Data are the mean ± SEM. The ratio was significantly changed over time in the Ad*COCH*GFP-transduced segments (RMANOVA, $P = 0.001$), whereas that of the AdGFP-transduced segments was not changed ($P > 0.05$).

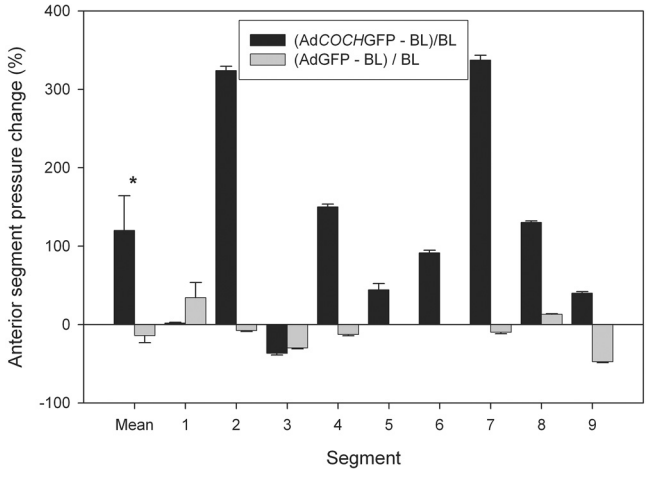

FIGURE 6. Change in ASP for individual Ad*COCH*GFP ($n = 9$)- and AdGFP ($n = 7$)-treated MOCAS 12 days after transduction (8 days after transduction in segment 1 and 11 days after transduction in segment 9). Data are expressed as the percentage change in ASP compared with BL. The mean ratio was subjected to a two-tailed paired *t*-test; *$P < 0.05$.

imately 40% after 5 to 9 days. Cochlin induction by TGFβ2 was slower, suggesting that more complex interactions may be involved in the ASP elevation compared with that after *COCH* transduction alone. In contrast, OF gradually increased with time in control AdGFP-transduced MOCAS. There is normally a gradual increase in OF and a decrease in ASP over several days that is probably due to washout of ECM and/or increased permeability of the thin sclera.[23,35] Future investigations may unravel further details of the processes involved in IOP and ASP elevation.

The intersegment variability in the OF changes and ASP in response to cochlin expression also suggests that differences in cochlin levels within the tissue and in cochlin–protein interactions in different TM segments play a role. We identified annexin A2 as a molecule that may interact with cochlin in the glaucomatous TM.[15] Annexin A2 is a membrane binding protein that has been implicated to play a role in exocytosis.[36] However, not all functions of annexin A2 are known and its role in the TM remains unclear. The role of annexin A2 in dynamic remodeling of the cytoskeleton,[37] may be linked to the cochlin-annexin A2 interaction in the TM. The presence of cochlin may also contribute to collagen degradation,[9] leading to alteration of TM ECM and consequently to modulation of OF. However, at present there is no direct evidence that cochlin expression affects collagen degradation in vivo.

The observed effect of cochlin is not explicable simply due to overexpression of cochlin causing clogging of the TM. In biological systems, more than a 15% variation in total protein expression under only slightly different conditions (for example, circadian fluctuations) is not uncommon, and it is unlikely that overexpression of a single protein would exceed the same level of the natural variation in total protein expression. Perfusion experiments performed using human organ culture anterior segments (HOCASs) with BSA and myocilin also suggest that expression of a single protein alone does not always lead to blockage of the outflow pathway. Expression of myocilin alone, even at a much higher protein level of 25 μg/mL, was insufficient to decrease OF in HOCAS unless aqueous humor was also present.[38,39]

The present study also suggests that *COCH* gene delivery to the TM may be used to develop a glaucoma model in vivo. However, it is not known whether transient cochlin expression in the TM and secretion into the aqueous humor is sufficient to maintain permanent changes in OF and IOP. If prolonged cochlin expression is necessary to maintain IOP elevation and OF reduction in vivo, transgene delivery by lentivectors (Buie LK, et al. *IOVS* 2007;48:ARVO E-Abstract 2050)[40] and scAAV vectors[33] should be considered, since adenoviruses, such as those used in the present study, provide only transient gene delivery.[41,42] Further investigations are needed to elucidate the biology of the cochlin effect on OF and IOP and perhaps reveal approaches that may be developed for glaucoma therapy.

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